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Insertion of a Bacterial Secondary Transport Protein in the Endoplasmic Reticulum Membrane*

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The sodium ion-dependent citrate carrier of *Klebsiella pneumoniae* (CitS) contains 12 hydrophobic potential transmembrane domains. Surprisingly, an alkaline phosphatase fusion study in *Escherichia coli* has suggested that only 9 of these domains are embedded in the membrane, and 3 are translocated to the periplasm (van Geest, M., and Lolkema, J. S. (1996) *J. Biol. Chem.* 271, 25582–25589). To provide independent data on the topology and mode of membrane insertion of CitS, we have investigated its insertion into the endoplasmic reticulum (ER) membrane. By using *in vitro* translation of model proteins in the presence of dog pancreas microsomes, each of the putative transmembrane segments of CitS was assayed for its potency to insert into the ER membrane, both as an isolated segment as well as in the context of COOH-terminal truncation mutants. All 12 segments were able to insert into the membrane as N_{cyt}-C_{lum} signal anchor sequences. In a series of COOH-terminal truncation mutants, the segments inserted in a sequential way except for one segment, segment Vb, which was translocated to the lumen. Hydrophobic segments VIII and IX, which, according to the alkaline phosphatase fusion study, are in the periplasm of *E. coli*, form a helical hairpin in the ER membrane. These observations suggest a topology for CitS with 11 transmembrane segments and also demonstrate that the sequence requirements for signal anchor and stop transfer function are different.

Multispanning integral membrane proteins consist of bundles of hydrophobic α-helices which are oriented more or less perpendicularly to the plane of the membrane. A critical step in the biosynthesis of membrane proteins is their insertion into the lipid bilayer. In the endoplasmic reticulum (ER),[1] insertion of membrane proteins into the membrane and preprotein translocation into the lumen proceed by similar mechanisms (for review, see Refs. 1 and 2). The process of targeting membrane and secretory proteins begins in the cytosol when a hydrophobic segment of a nascent polypeptide chain, either a signal sequence or the first transmembrane segment, emerges from the ribosome and is recognized by the signal recognition particle (SRP). The binding of SRP to the ribosome results in a translational arrest, and the whole complex is targeted to translocation sites in the membrane via interactions with the SRP receptor. Translation resumes after dissociation of SRP from the ribosome, and the nascent chain is transferred cotranslationally into the translocon. The central component of the translocon is the heterotrimeric Sec61p complex (for review, see Refs. 3 and 4). Because the Sec61p channel both translocates secretory proteins and integrates membrane proteins into the lipid bilayer, it must coordinate a number of different functions. In the case of membrane proteins, cytoplasmic domains have to be left in the cytoplasm, lumenal domains have to be passed through the membrane, and transmembrane domains have to be properly oriented and inserted into the membrane.

In the most simple view, membrane integration of polytopic membrane proteins is thought to be directed by series of alternating signal anchor (SA) and stop transfer (ST) sequences (5–7). A SA sequence is defined as a hydrophobic segment that has the ability to insert into the membrane in a N_{cyt}-C_{lum} orientation. The sequence following the SA will be translocated to the lumen until translation of the next hydrophobic region, the ST sequence, which is retained in the membrane as a N_{lum}-C_{cyt} transmembrane segment. The following sequences will have a cytoplasmic location until the next SA sequence emerges from the ribosome, and the cycle starts all over again. Although the assembly of some membrane proteins in the ER appears to occur via this mechanism, many studies have suggested that the insertion process is often more complicated and depends on the coordinate action of two or more topogenic sequences in the nascent chain (8–10). A role for the ribosome in the recognition of a transmembrane segment has also been documented (11–13).

In *Escherichia coli*, utilization of the same pathway for export of preproteins and insertion of membrane proteins is a matter of debate. It has been shown that *E. coli* contains essential genes coding for the proteins Ffh and FtsY, which are homologous to subunits of the eukaryotic SRP and SRP receptor proteins (14, 15). Evidence is accumulating that these proteins play an important role in the assembly of inner membrane proteins, suggesting a pathway similar to the one in the ER (16–18). Structural similarities between the *E. coli* SecYEG complex and the eukaryotic Sec61p complex suggest that in *E. coli*, after targeting, membrane insertion may proceed via the Sec machinery.

Topology studies of several engineered model membrane proteins (19) and multispanning membrane proteins in which internal transmembrane segments were deleted (20–22) have

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[1] The abbreviations used are: ER, endoplasmic reticulum; SRP, signal recognition particle; SA, signal anchor; ST, stop transfer; CitS, citrate carrier of *K. pneumoniae*; PhoA, alkaline phosphatase; Endo H, endoglycosidase H; SPase, signal peptidase; Lep, leader peptidase; LC, Lep-CitS.

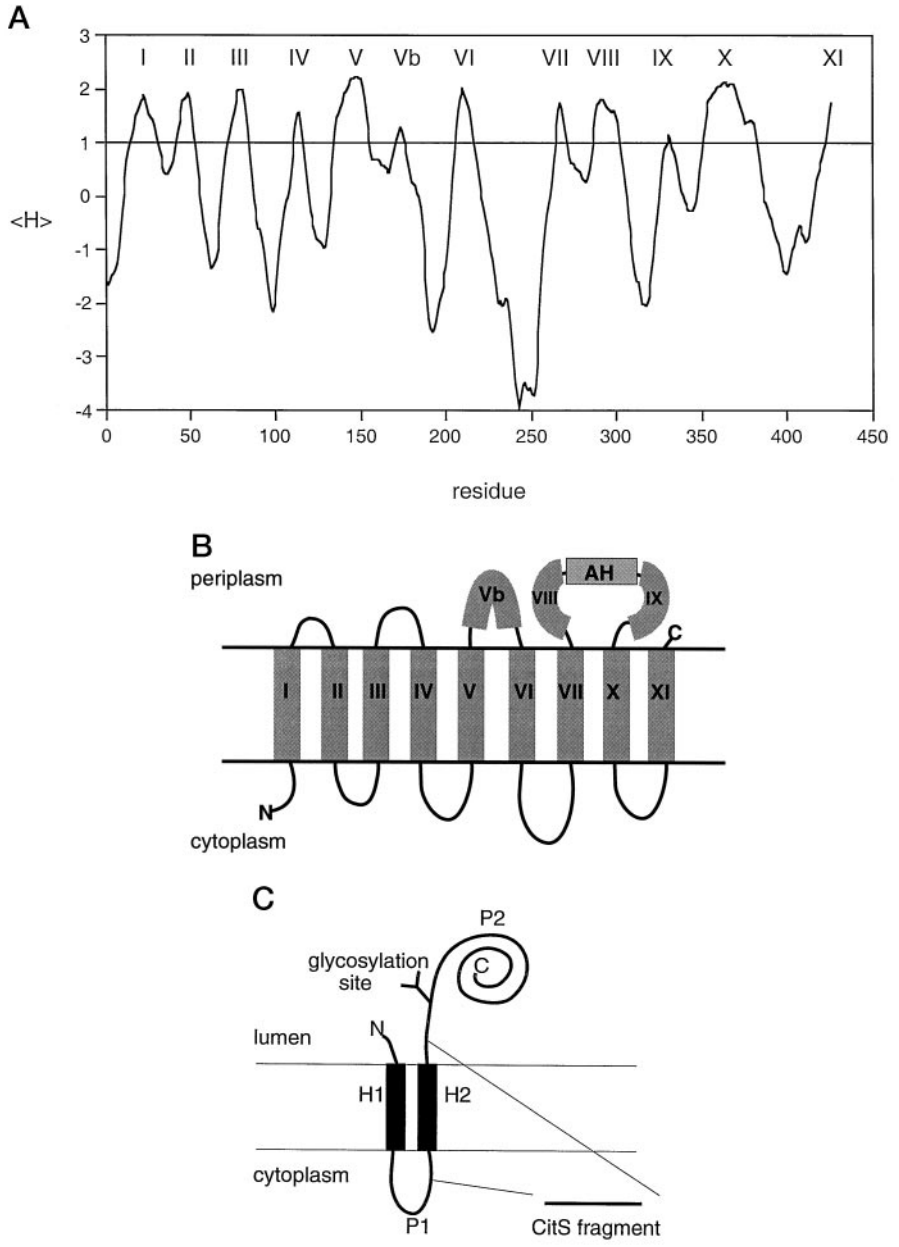

FIG. 1. **Membrane topology model of CitS in the *E. coli* membrane and of a modified Lep molecule in the microsomal membrane.** *Panel A*, hydrophobicity plot for CitS using TOPPRED with default parameter settings (38). The *horizontal line* indicates the cutoff for "certain" transmembrane segments. *Panel B*, model for the membrane topology of CitS based on CitS-PhoA fusions (23). The 12 hydrophobic segments predicted to be transmembrane from the hydropathy profile are indicated by I–V, Vb, and VI–XI. Segment AH is strongly amphipathic when folded as an α-helix. *Panel C*, membrane topology of Lep. Transmembrane segments H1 and H2 are connected by a short cytoplasmic loop P1. The large periplasmic domain P2 is translocated across the ER membrane. A glycosylation site is introduced 15 amino acids downstream of the COOH-terminal end of H2. Engineered sites in the coding region of Lep allow the exchange of H2 for CitS fragments.

demonstrated that in the bacterial system also, insertion in the cytoplasmic membrane is not always a strictly sequential process. Recently, the membrane topology of the sodium ion-dependent citrate carrier of *Klebsiella pneumoniae* (CitS) in the *E. coli* cytoplasmic membrane was studied using a series of CitS-PhoA fusions (23). Hydropathy analysis of the sequence revealed that the polypeptide contains 12 hydrophobic segments that might span the membrane (Fig. 1*A*), but the biochemical data unexpectedly suggested the presence of only 9 transmembrane segments. In this model, 3 hydrophobic segments are excluded from the membrane (Fig. 1*B*), suggesting that during insertion of CitS factors other than hydrophobicity may play a role in the recognition of transmembrane segments.

In view of the unexpected results from the PhoA fusion analysis, we have sought to obtain independent evidence for or against the 9 transmembrane model. To this end, an extensive topological analysis of CitS has been carried out using *in vitro* translation in the presence of dog pancreas microsomes. The goal has been to identify the topogenic signals and to determine whether they insert as independent units or whether the insertion potencies of the topogenic sequences are context-dependent. Interestingly, our analysis provides clear evidence for a topology with 11 transmembrane segments, which fits better with the hydrophobicity analysis results than does the previous 9 transmembrane model. We also find that a marginally hydrophobic segment in CitS works well as an internal SA sequence but does not have ST activity, suggesting that SA and ST sequences may be recognized in different ways by the translocon.

MATERIALS AND METHODS

Enzymes and Chemicals—Unless stated otherwise, all enzymes needed for the *in vitro* transcription and translation reactions, as well as plasmid pGEM1 were obtained from Promega Biotech (Madison, WI). The cap analog m7G(5′)-ppp(5′) and [^{35}S]methionine were obtained from Amersham Pharmacia Biotech (Uppsala, Sweden). Endo H was purchased from Boehringer Mannheim. The glycosylation acceptor peptide *N*-benzoyl-Asn-Leu-Thr-*N*-methylamide and the nonacceptor peptide *N*-benzoyl-Asn-Leu-(allo)Thr-*N*-methylamide were synthesized according to Erickson and Merrifield (24). The signal peptidase (SPase) inhibitor *N*-methoxysuccinyl-Ala-Ala-Pro-Val-chloromethyl ketone was obtained from Sigma. Oligonucleotides were purchased from Kebo Laboratory (Stockholm, Sweden) and from Eurosequence (Groningen, The Netherlands).

TABLE I
Insertion efficiency of the individual CitS fragments in the ER membrane

Insertion efficiency was measured as the degree of glycosylation of Lep constructs in which the H2 domain was replaced by the indicated CitS fragments (see also Fig. 1*B*).

Construct	CitS residues	Average hydrophobicity[a]	Net charge		Glycosylation
			P1 loop	P2 loop[b]	
					%
Lep			6+	0	80
LCI	1–47	0.73	7+	1−	75
LCII	45–74	0.79	6+	0	60
LCIII	74–106	0.83	7+	0	69
LCIV	104–142	0.60	6+	2+	78
LCV	141–175	0.92	6+	1−	60
LCVb	173–195	0.56	6+	1−	79
LCVI	201–240	0.84	6+	1+	78
LCVII	264–287	0.59	6+	1−	90
LCVIII	286–321	0.75	8+	2−	91
LCAH	310–331	0.06			0
LCIX	334–357	0.54	7+	1−	66
LCX	365–389	0.86	6+	2−	72
LCXI	401–446	0.62	6+	1−	88

[a] Calculated for the 20-residue-long predicted transmembrane segment in the CitS fragment using the Eisenberg scale (39).
[b] Measured from the COOH-terminal end of the predicted transmembrane segment to the glycosylation site in P2.

DNA Manipulation Techniques—All DNA manipulations were performed using *E. coli* strains MC1061 (25) and JM110 (26). Plasmid pGEM1 carrying a modified *lep* gene as an *Xba*I-*Sma*I fragment behind the SP6 promoter (27) was used for the construction of different Lep-CitS (LC) constructs. In the modified *lep* gene the nucleotide sequences coding for the Asn-Glu-Thr glycosylation acceptor site at position 214–216 in the wild type protein was changed to the nonacceptor sequence Gln-Gln-Thr, whereas an Asn-Ser-Thr glycosylation acceptor site was introduced at codons 90–92, 15 amino acids downstream of H2 (see Fig. 1*C*). Furthermore *Bcl*I and *Nde*I restriction sites were introduced flanking the H2 region, in codons 59 and 80, respectively. Different fragments of the CitS gene were amplified by polymerase chain reaction from pKSCitS (23) using forward primers containing a *Bcl*I restriction site and backward primers containing an *Nde*I restriction site. After purification, the polymerase chain reaction products were digested with the *Bcl*I and *Nde*I restriction enzymes and ligated into the pGEM1-Lep plasmid digested with the same two enzymes (see also Fig. 1*C*).

A second modified *lep* gene in plasmid pGEM1 was used to measure ST efficiency of CitS sequences and has been described by Sääf *et al.* (28). Briefly, this modified *lep* gene contains an *Spe*I site and a *Bgl*II site engineered in codons 226/227 and 231/232, respectively. The original glycosylation site of the wild type gene was removed as described above, and two new potential glycosylation sites were introduced around codons 97 and 259, respectively. CitS sequences were amplified with forward primers containing an *Spe*I site and backward primers containing a *Bam*HI site (compatible with *Bgl*II) and were cloned between the *Spe*I and *Bgl*II sites of the modified *lep* gene (see also Fig. 4*C*). All constructs were confirmed by sequencing the plasmid DNA.

In Vitro Transcription/Translation in Reticulocyte Lysate—The pGEM1 plasmids carrying the relevant constructs were linearized at an *Sma*I site immediately downstream of the coding region before *in vitro* transcription. mRNA was transcribed from the SP6 promoter in the following reaction mixture: 4 μl of 5 × SP6 transcription buffer, 4 μl of rNTPs +Gcap mix (25 mM ATP, CTP, UTP, 15 mM GTP, 10 mM Gcap), 2 μl of SP6 enzyme mix, and 10 μl of purified and linearized plasmid DNA containing about 0.5 μg of DNA. Transcriptions were carried out at 30 °C for 12 h. The synthesized mRNAs were purified by using a Rneasy Minikit (Qiagen).

In vitro translation of [^{35}S]methionine-labeled proteins from the *in vitro* synthesized mRNA was performed in reticulocyte lysate in the presence or absence of dog pancreas microsomes as described by Liljeström *et al.* (29). Translation reactions without microsomes contained 0.1% of the detergent pentaethylene glycol monooctyl ether (Fluka). Translation was carried out at 30 °C for 1 h. Translocation of the P2 domain to the lumenal side of the microsomes was assayed by the appearance of *N*-linked glycosylation. For some constructs, the assay was repeated in the presence of a glycosylation acceptor tripeptide and a nonacceptor tripeptide as described (30), or in the presence of SPase inhibitor.[2] For construct LCVb, glycosylation was assayed by Endo H treatment of the translation mixture. The mixtures was incubated with 50 milliunits/ml Endo H in a 1% SDS, 50 mM sodium citrate buffer, pH 6.0, for 6 h at 37 °C. All samples were analyzed by SDS-polyacrylamide gel electrophoresis using 10% or 12% polyacrylamide gels. The gels were scanned in a FUJIX Bas 1000 PhosphorImaging Plate Scanner and analyzed using the MacBAS software (version 2.31).

[2] M. Nusier and M. O. Lively, unpublished data.

RESULTS

Putative Transmembrane Segments of CitS—A study in *E. coli* with a set of COOH-terminally truncated CitS molecules to which the topological reporter protein alkaline phosphatase was fused (PhoA fusions) suggested that the CitS molecule spans the membrane nine times (23). The topology model derived from the PhoA fusion data (Fig. 1*B*) is characterized by a periplasmic COOH terminus and two large, relatively hydrophobic loops in the periplasm which contain segments Vb, VIII, and IX, which were predicted to be transmembrane. Hydrophobic segments VIII and IX are separated by a region AH that could potentially form a strongly amphiphilic α-helix. The different segments of CitS and their average hydrophobicities are listed in Table I.

The first objective of this study was to examine systematically the propensity of the 12 predicted transmembrane segments plus the putative amphipathic helix AH to form individually a transmembrane α-helix in the ER membrane using an *in vitro* translation/translocation system. The *E. coli* inner membrane protein leader peptidase (Lep) was used as an insertion vehicle for the CitS fragments. Lep is anchored in the cytoplasmic membrane by two NH_2-terminal transmembrane segments H1 and H2 which are connected by the P1 domain. The catalytic P2 domain forms the COOH-terminal half of the protein (Fig. 1*C*). Upon *in vitro* transcription/translation in the presence of dog pancreas microsomes the Lep molecule inserts into the microsomes with both the NH_2 and COOH termini facing the lumen and with the P1 and P2 domains in the cytoplasm and lumen, respectively. An engineered glycosylation site COOH-terminal of H2 is glycosylated efficiently upon correct insertion into the microsomal membrane. Glycosylation of the molecule results in an increase in molecular mass of about 2 kDa relative to the observed molecular mass in the absence of microsomes. The efficiency of glycosylation of Lep under standard conditions is 80–90% (30–33).

SA Function of Individual CitS Segments—The H2 segment of Lep was exchanged with the different segments of CitS (Fig. 1*C*). CitS segments with SA function will result in glycosylation of the P2 domain, whereas a segment that lacks SA function will result in nonglycosylated molecules. In all cases but one

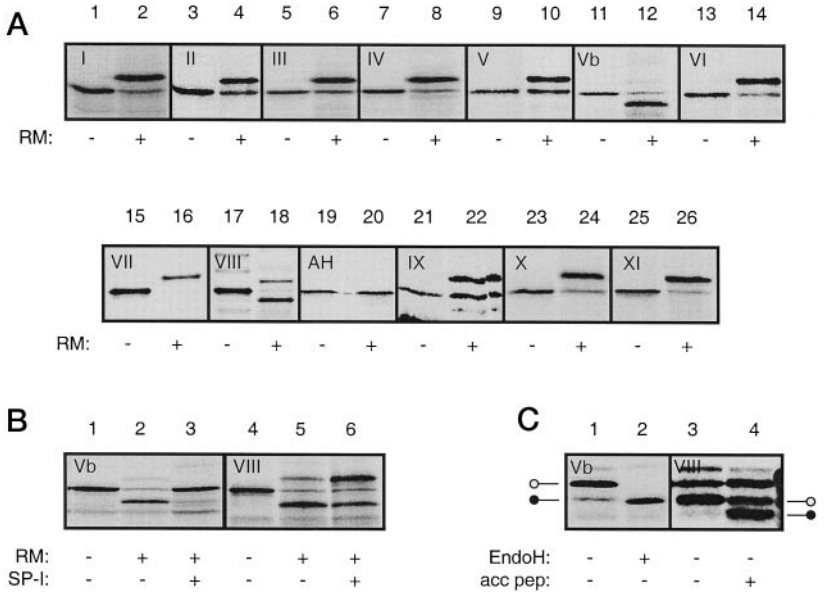

FIG. 2. **All 12 hydrophobic segments of CitS insert individually into the microsomal membrane.** *Panel A*, SDS-gel analysis of the following constructs expressed *in vitro* in reticulocyte lysate in the absence (−) and in the presence (+) of rough microsomes (*RM*): LCI (*lanes 1* and *2*), LCII (*lanes 3* and *4*), LCIII (*lanes 5* and *6*), LCIV (*lanes 7* and *8*), LCV (*lanes 9* and *10*), LCVb (*lanes 11* and *12*), LCVI (*lanes 13* and *14*), LCVII (*lanes 15* and *16*), LCVIII (*lanes 17* and *18*), LCAH (*lanes 19* and *20*), LCIX (*lanes 21* and *22*), LCX (*lanes 23* and *24*), and LCXI (*lanes 25* and *26*). The *number* in the name of the construct refers to the segment of CitS which replaces the H2 domain of Lep (see Fig. 1*B*). The results are summarized in Table I. *Panel B*, translation products from *in vitro* translation of constructs LCVb (*lanes 1–3*) and LCVIII (*lanes 4–6*) in the absence and presence of SPase inhibitor (*SP-I*). *Panel C*, Endo H treatment of LCVb translated in the presence of RM (*lanes 1* and *2*) and translation of LCVIII in the presence of RM and in the presence of a glycosylation acceptor tripeptide (*acc pep*, *lane 4*) or a nonacceptor tripeptide (*lane 3*). ○, glycosylated products; ●, nonglycosylated products.

(LC-AH), translation in the presence of the microsomes resulted in a molecular mass that was about 2 kDa larger than that observed in the absence of the microsomes (Fig. 2*A* and Table I), demonstrating a transmembrane disposition of all of the CitS fragments except the amphipathic AH segment. Constructs LCVb and LCVIII were partly processed to a slightly smaller product when translated in the presence of microsomes (*lanes 12* and *18*). To test the possibility that in these constructs the P2 domain is removed after translocation to the lumen by the endogenous SPase activity, insertion into the microsomal membrane was performed in the presence of *N*-methoxysuccinyl-Ala-Ala-Pro-Val-chloromethyl ketone, an inhibitor of the mammalian SPase.[2] Formation of the proteolytic product in LCVb and LCVIII was largely inhibited under these conditions, indicating translocation of the P2 domain by segments Vb and VIII (Fig. 2*B*). For construct LCVIII, the amount of full-length glycosylated protein was increased significantly, but, surprisingly, for construct LCVb the full-length protein was not glycosylated. Evidence for the glycosylation of the cleaved form of construct LCVb was obtained by treatment with Endo H, an enzyme that removes *N*-linked oligosaccharides (Fig. 2*C*, *lanes 1* and *2*). Apparently, construct LCVb is glycosylated only after cleavage by SPase. Similarly, glycosylation of construct LCVIII could be demonstrated by a reduction in the molecular mass of the processed product by about 2 kDa when translation was performed in the presence of a glycosylation acceptor tripeptide, a competitive inhibitor of *N*-linked glycosylation, but not in the presence of a nonacceptor tripeptide (Fig. 2*C*, *lanes 3* and *4*).

These results demonstrate that all 12 hydrophobic segments of CitS can function as internal SA sequences. Strikingly, the 3 hydrophobic segments (Vb, VIII, and IX) that are postulated to be translocated across the membrane in the PhoA model (Fig. 1*B*) are SA sequences that are equally as efficient as the other segments.

SA and ST Function of COOH-terminally Truncated CitS Fragments—The H2 segment of Lep was replaced by CitS fragments of increasing length, and the membrane topology was assessed by glycosylation of the Lep P2 domain (Table II). Extending the CitS fragment containing hydrophobic segment I with segment II (construct LCI-II) resulted in nonglycosylated molecules (Fig. 3*A*). Apparently, segment II is an efficient ST sequence (Fig. 3*B*). Addition of segment III resulted again in glycosylated molecules (construct LCI-III, Fig. 3*A*), consistent with the formation of three transmembrane segments. Similarly, successive addition of segments IV and V resulted in nonglycosylated and glycosylated molecules, respectively, suggesting topologies with four and five transmembrane segments.

Extension of the CitS fragment with the next hydrophobic segment, Vb, did not result in the formation of an additional transmembrane segment as evidenced by a high degree of glycosylation of construct LCI-Vb. Apparently, segment Vb does not have ST activity but instead is translocated to the lumen. This result is in accord with the topology study of CitS in *E. coli* (Fig. 1*B*); fusion of PhoA to the COOH-terminal end of fragment I-Vb resulted in a periplasmic location of the PhoA moiety.

As expected, construct LCI-VI was not glycosylated, indicating that one additional transmembrane segment is formed and that, most likely, segment Vb remains in the lumen. Addition of segment VII (LCI-VII) gave rise to efficiently glycosylated molecules, consistent with a total of seven transmembrane segments. Up to this point, the results are thus in full agreement with the PhoA topology model.

Constructs LCI-VIII and LCI-IX (including the AH segment) were nonglycosylated and glycosylated, respectively, showing that in the ER membrane segments VIII and IX form a pair of transmembrane helices. This result is different from the PhoA studies in *E. coli*, where PhoA fusions between segments VII and X all resulted in periplasmic PhoA molecules. Finally, addition of segments X and XI (LCI-X and LCI-XI) resulted in nonglycosylated and glycosylated molecules, respectively, indicating that segments X and XI both span the membrane.

We conclude that CitS spans the microsomal membrane 11

TABLE II
Membrane topology of CitS fragments in the ER membrane
The glycosylation of Lep constructs in which the H2 domain was replaced by the indicated CitS fragments was measured.

Construct	CitS residues	Glycosylation	Construct	CitS residues	Glycosylation	Construct	CitS residues	Glycosylation	Localization
		%			%			%	
LCI	1–46	75							Lumenal
LCI-II	1–74	0							Cytoplasmic
LCI-III	1–105	85							Lumenal
LCI-IV	1–142	0							Cytoplasmic
LCI-V	1–175	60							Lumenal
LCI-Vb	1–195	77							Lumenal
LCI-VI	1–240	0							Cytoplasmic
LCI-VII	1–287	78	LCVII	264–287	90				Lumenal
LCI-VIII	1–321	0	LCVII-VIII	264–321	13				Cytoplasmic
LCI-IX	1–357	55	LCVII-IX	264–357	52	LCIX	334–357	66	Lumenal
LCI-X	1–389	0	LCVII-X	264–389	18	LCIX$\hat{X}$	334–389	18	Cytoplasmic
LCI-XI	1–446	72	LCVII-XI	264–446	n.d.	LCIX$\hat{X}$I	334–446	60	Lumenal

times and has one relatively hydrophobic loop (Vb) in the lumen (Fig. 3*C*).

SA and ST Function of Internal CitS Fragments—A series of Lep-CitS constructs was made to test the SA and ST function of various combinations of internal hydrophobic segments (Table II). Construct LCVII-VIII was not glycosylated, indicating that segments VII and VIII insert as a pair of transmembrane helices. The addition of segment IX (LCVII-IX) resulted in glycosylated molecules, consistent with the presence of three transmembrane segments. Successive addition of segments X and XI resulted in nonglycosylated and glycosylated molecules, respectively, again demonstrating membrane insertion of segments X and XI.

Similarly, the insertion of the three COOH-terminal hydrophobic segments of CitS in the absence of the NH_2-terminal part of the protein was studied. A construct containing segments IX and X (LCIX-X) was not glycosylated, indicating the formation of two transmembrane helices (Table II). As expected, the addition of segment XI gave rise to efficiently glycosylated molecules. In summary, successively adding the hydrophobic segments starting at segment VII or segment IX resulted in alternating glycosylated and nonglycosylated molecules in the same way as was observed in the presence of the NH_2-terminal parts. Thus, COOH-terminal fragments insert independently of the upstream transmembrane segments.

Exclusion of Segment Vb from the Membrane—Segments V, Vb, and VI are each able to function as SA sequences when replacing H2 in Lep (Table I). On the other hand, segment Vb does not function as a ST sequence in the context of the COOH-terminally truncated constructs (Fig. 3). Similarly, a CitS fragment containing segments V and Vb (LCV-Vb) resulted in glycosylation of the P2 domain (Fig. 4*A*, *lanes 1* and *2*). Because construct LCVb is partially cleaved by SPase whereas LCV is not (Fig. 2), the absence of SPase cleavage of LCV-Vb strongly suggests that it is segment V rather than Vb which spans the membrane in this construct. The addition of segment VI (LCV-VI) resulted in a cytoplasmic localization of the P2 domain (Fig. 4*A*, *lanes 3* and *4*). The results are the same as observed in the presence of the four NH_2-terminal hydrophobic segments and indicate that the presence of segment V at the NH_2-terminal side of Vb is enough to mediate the exclusion of Vb from the membrane, *i.e.* that Vb does not function as a ST sequence (Fig. 4*B*).

As a further test of the ST activity of segment Vb, this segment was inserted in a different Lep construct designed to measure ST efficiency directly (28). This construct consists of the entire Lep molecule with two engineered glycosylation sites flanking two unique restriction sites in the P2 domain. If a segment encoding a ST sequence is cloned between the two glycosylation sites, only one of the two sites will be glycosylated; if the segment lacks ST activity, both sites will be modified (Fig. 4*C*). Insertion of segment Vb between the two glycosylation sites resulted in mostly double-glycosylated molecules (Fig. 4*A*, *lanes 7* and *8*), again demonstrating that segment Vb does not function efficiently as a ST sequence.

To test whether the results obtained with segment Vb are related to the relatively low hydrophobicity of this fragment (Table I) the experiments were repeated with segment IX, which also has a relatively low hydrophobicity. Segment VIII is an efficient SA sequence (Table I and Fig. 2). The addition of segment IX to VIII (LCVIII-IX) resulted in a low percentage of glycosylated molecules (Fig. 5*A*, *lanes 1* and *2*). Thus, in contrast to segments V and Vb, segments VIII and IX form a pair of transmembrane helices. When tested in the ST Lep construct, segment IX also displayed a much higher ST efficiency (*i.e.* a higher fraction of single-glycosylated molecules) than observed with fragment Vb (Fig. 5*A*, *lanes 7* and *8*).

To test further whether the ST activity of segments Vb and IX is influenced by the NH_2-terminal sequence preceding the segments, segments Vb and IX were exchanged in constructs LCV-Vb and LCVIII-IX resulting in the constructs LCVIII+Vb and LCV+IX. Also in the context of segment VIII, segment Vb does not exhibit any ST activity as indicated by a high percentage of glycosylated molecules (77%, Fig. 4*A*, *lanes 5* and *6*). Construct LCV+IX resulted in 40% glycosylated molecules, indicating that the ST activity of segment IX in this construct was significantly lower than in construct LCVIII-IX (Fig. 5, *lanes 5* and *6*).

A somewhat unexpected result was obtained when the fragment replacing H2 containing segments VIII and IX was extended with segment X. In this context, segment X apparently does not function as a SA sequence, as evidenced by the almost complete lack of glycosylation of the LCVIII-X construct (Fig. 5*A*, *lanes 3* and *4*). It should be noted, though, that segments VIII and IX are inserted in the opposite orientation compared with the wild type CitS in this construct.

DISCUSSION

The CitS polypeptide contains 12 hydrophobic domains that are long and hydrophobic enough to be considered potential transmembrane helices. A PhoA fusion study in *E. coli* suggested that only 9 of these domains span the membrane, and 3 hydrophobic domains are located in the periplasm (23). Such a topology would indicate a mechanism of insertion in the membrane more complex than a simple sequential insertion of the hydrophobic segments. In view of this result, we have sought to provide independent data on the topology and mode of insertion of CitS by studying its assembly into microsomal membranes.

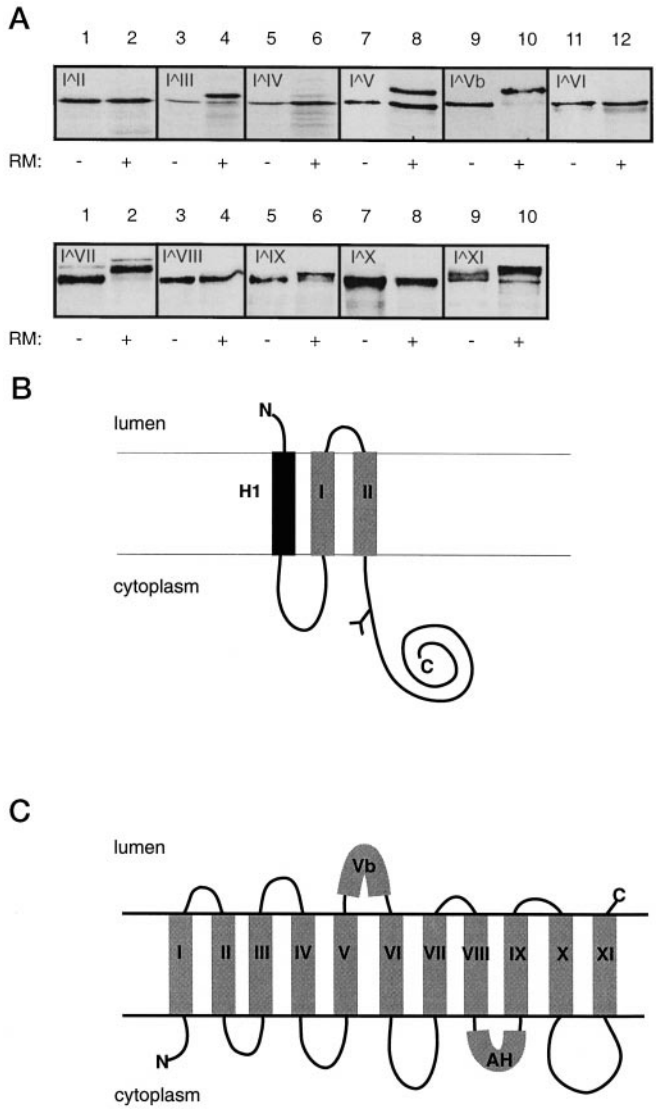

FIG. 3. **Replacement of the H2 domain of Lep by CitS fragments with an increasing number of transmembrane segments.** *Panel A*, SDS-gel analysis of translation products from *in vitro* translation in the absence (−) and in the presence (+) of rough microsomes (*RM*) of the following constructs: LCI-II (*lanes 1* and *2*), LCI-III (*lanes 3* and *4*), LCI-IV (*lanes 5* and *6*), LCI-V (*lanes 7* and *8*), LCI-Vb (*lanes 9* and *10*), LCI-VI (*lanes 11* and *12*), LCI-VII (*lanes 13* and *14*), LCI-VIII (*lanes 15* and *16*), LCI-IX (*lanes 17* and *18*), LCI-X (*lanes 19* and *20*), and LCI-XI (*lanes 21* and *22*). In the constructs the *numbers* before and after-refer to the first and last hydrophobic segment in the CitS fragment, respectively. The results are summarized in Table II. *Panel B*, topology of a Lep-CitS construct (construct LCI-II) in which the H1 domain of Lep (*black rectangle*) is followed by the first two hydrophobic segments of CitS (*gray rectangles*). *Panel C*, topology model of CitS in the microsomal membrane suggested by the glycosylation data in Table II.

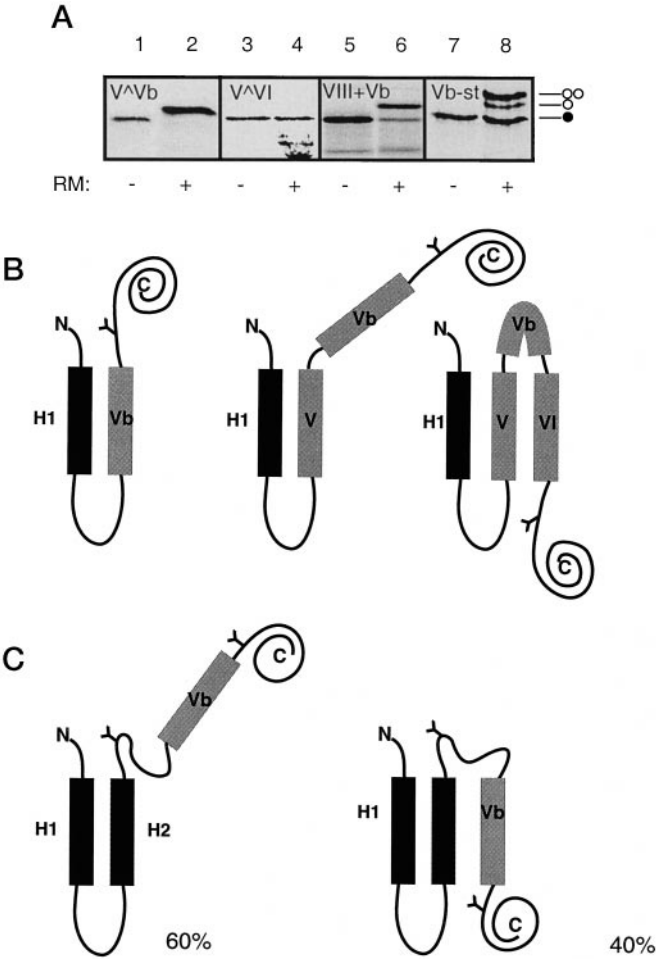

FIG. 4. **Exclusion of segment Vb from the microsomal membrane.** *Panel A*, SDS analysis of translation products from *in vitro* translation in the absence (−) and presence (+) of rough microsomes (*RM*) of constructs LCV-Vb (*lanes 1* and *2*), LCV-VI (*lanes 3* and *4*), LCVIII+Vb (*lanes 5* and *6*), and the Lep construct carrying segment Vb in the P2 domain as depicted in *panel C* (*lanes 7* and *8*). ST activity of segment Vb when placed in the P2 domain results in single-glycosylated molecules, whereas an inability to function as a ST sequence results in double-glycosylated molecules. ●, nonglycosylated molecules; ○, single-glycosylated molecules; ○○, double-glycosylated molecules. *Panel B*, predominant topologies of constructs LCVb, LCV-Vb, and LCV-VI as suggested by the glycosylation data in Fig. 2 and Fig. 4*A*. *Panel C*, modified Lep protein used to measure the stop transfer activity of segment Vb. The protein consists of the entire Lep molecule (H1 and H2 are depicted as *black rectangles*) and allows the insertion of a sequence into the middle of the P2 domain. Indicated are the two engineered glycosylation sites around the inserted Vb fragment (*gray rectangle*). The ratio of the observed topologies is indicated.

We have thus assayed the SA and ST function of each putative transmembrane segment, both as an individual fragment and in COOH- and NH_2-terminally truncated CitS molecules.

The different CitS fragments were introduced in place of the H2 transmembrane segment of Lep, and glycosylation of the P2 domain (see Fig. 1*C*) was used as an indicator of membrane insertion. The insertion of Lep into the microsomal membrane has been studied extensively (31–33). It has been shown that the H1 domain has an intrinsic ability to target to the ER membrane and to insert with the NH_2 terminus in the lumen and that the orientation of the H1 segment is largely determined by the highly positively charged P1 loop. Because all the Lep-CitS constructs contain the H1 domain and the P1 loop, targeting the molecules to the membrane is mediated by H1, and the NH_2-terminal end of the CitS fragment is located in the cytoplasm. Thus, we can study the "intrinsic" SA and ST function of various CitS fragments in molecules that have already been targeted to the microsomal membrane. In this respect, our approach differs from similar studies in which potential transmembrane domains are inserted in constructs without a preceding ER targeting sequence (10, 34–36) where both targeting and translocation are assayed.

All 12 hydrophobic domains of CitS were able to insert into the ER membrane when replacing the H2 domain of Lep, irrespective of their orientation in the wild type CitS protein. The SA activity of the hydrophobic domains given as percentage of glycosylation varied between 60 and 91% (Table I). There is no obvious correlation between the hydrophobicity of the

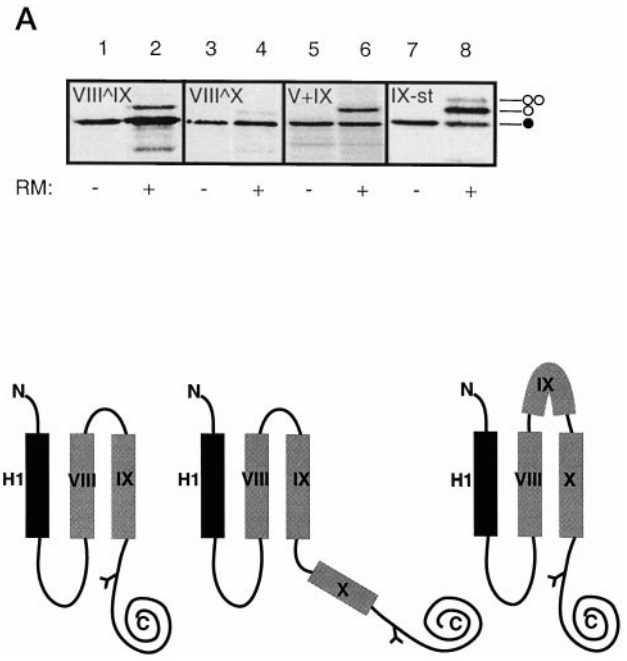

FIG. 5. **Insertion of segment IX.** *Panel A*, SDS-gel analysis of translation products after *in vitro* translation in the absence (−) and presence (+) of rough microsomes (*RM*) of constructs LCVIII-IX (*lanes 1* and *2*), LCVIII-X (*lanes 3* and *4*), LCV+IX (*lanes 5* and *6*), and the Lep construct carrying segment IX in the P2 domain (*lanes 7* and *8*) as explained in Fig. 4*C*. ●, nonglycosylated molecules; ○, single-glycosylated molecules; ○○, double-glycosylated molecules. *Panel B*, topology of LCVIII-IX and possible topologies of construct LCVIII-X.

segment and the degree of glycosylation. Similarly, the presence of additional positive or negative charges at the COOH-terminal side of the CitS fragment does not seem to affect SA function in any regular way (Table I). Most likely this is because of the highly positively charged P1 loop forcing the CitS segment to adopt a N_{cyt}-C_{lum} orientation.

A simple sequential insertion mechanism would predict that the individual hydrophobic segments insert one after the other in the membrane. Except for segment Vb, this was observed when segment I was successively extended with the following segments. Also, when segments VII and IX were extended with the downstream hydrophobic domains (Table II), a similar sequential insertion was observed, illustrating that NH_2-terminal fragments of CitS are not required for the insertion of COOH-terminal fragments and that in these constructs the overall orientation of the segments is simply determined by the most NH_2-terminal segment.

Interestingly, while fully active as a SA sequence, segment Vb does not function as an efficient ST sequence in any of the constructs tested. The low ST function of Vb correlates with its rather low average hydrophobicity. However, the high ST activity of segment IX, which has an even lower hydrophobicity (Table I), suggests that current hydrophobicity scales do not fully predict ST function or that the specific amino acid sequence of a segment may play a role in ST activity (28). In addition, the context of a segment seems to affect the ST function as well. Although segment Vb lacks detectable ST activity when preceded by segment V or VIII (Fig. 4*A*), it does exhibit low but significant ST activity when placed in the middle of the P2 domain. Similarly, the ST activity of segment IX is significantly lower in construct V+IX than observed in construct VIII-IX or in the P2 domain (Fig. 5*A*).

The insertion behavior of the CitS fragment comprising hydrophobic segments VIII, IX, and X demonstrates that the same fragment may insert differently in the membrane depending on the orientation. In the normal situation in the CitS molecule, the NH_2 terminus of segment VIII is on the lumenal side of the membrane, and the three segments insert sequentially (Fig. 3*C*). When the NH_2 terminus of segment VIII is forced to the cytoplasmic side, segment VIII functions as a SA sequence, and the addition of segment IX results in a pair of transmembrane helices (Fig. 5*B*). However, when segment X is added, the fragment adopts either one of the two topologies indicated in Fig. 5*B* with only two membrane spans.

The exclusion of segment Vb from the membrane and the unexpected topology of the LCVIII-X construct suggest that the topogenesis of a membrane protein is not determined simply by the presence of hydrophobic domains but is a controlled process in which the destination of a hydrophobic segment is influenced by the context of the segment in the protein and the orientation in the membrane, *i.e.* whether it acts as a SA or a ST sequence. Probably, interactions between different parts of the nascent chain and the ribosome and/or the translocation machinery (11–13) determine the destination of the hydrophobic segments.

The present study indicates that CitS traverses the microsomal membrane 11 times (Fig. 3*C*), whereas a study in *E. coli* suggested a model with only 9 transmembrane segments (Fig. 1*B*). Both studies make use of COOH-terminally truncated CitS molecules to which a topological marker was fused. Nevertheless, although in both models segment Vb is translocated across the membrane, segments VIII and IX form a transmembrane helix pair in the ER membrane but seem to have a periplasmic location in *E. coli*, despite the high hydrophobicity of segment VIII. Possibly, both models are correct, *i.e.* the bacterial and ER insertion machinery interpret the topological information in the nascent chain in a different way, resulting in two CitS proteins with different folding (see also Ref. 37). Alternatively, the two insertion machineries deal differently with the truncated proteins because different reporter molecules were used (PhoA or P2) or because in the bacterial system COOH-terminal sequences are required for proper insertion of segment VIII, whereas this would be less critical in the ER system. It should be noted that the model with 11 transmembrane segments conforms better to the hydrophobicity profile and to the so-called "positive inside" rule (32). Additional studies on the intact CitS protein are needed to resolve the contradiction between the results obtained in the *E. coli* and microsomal systems.

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